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**PROCESS FOR IDENTIFYING SUBSTANCES WHICH MODULATE THE ACTIVITY  
OF HYPERPOLARIZATION-ACTIVATED CATION CHANNELS**

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**BACKGROUND OF THE INVENTION**

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**Field of the Invention**

The present invention relates to the field of biological cell-to-cell communication and electrochemical signalling between biological cells. In particular, the present invention provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, and the use of this process.

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**Description of the Relevant Art**

Some genes of murine and human hyperpolarization-activated cation channels are already known. Examples include muHCN2(muHAC1) (Ludwig et al. (1998)), huHCN4 (Ludwig et al. (1999)), huHCN2 (Vaccari, T. et al. (1999) Biochim. Biophys. Acta 1446(3): 419-425), and those disclosed in WO 99/32615 and WO 99/42574. See, also, Tables 1-6 herein.

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Ludwig et al. (1998) have shown that muHCN2 can be transfected transiently in HEK293 cells, and that the corresponding channel in the transfected cells can be examined easily by electrophysiological methods (patch-clamp studies). The electrophysiological properties of the cloned channel correspond to the  $I_f$  or  $I_h$  current described in pacemaker cells, which had hitherto not been known on a molecular level (Ludwig et al. (1998), Biel et al. (1999)). The channel activates when the holding potential is changed toward hyperpolarization (potential at about B100 to B160 mV). However, the patch-clamp technique cannot be automated and is not suitable for high-throughput screening (HTS).

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Using suitable dyes, ion currents can be measured in an FLIPR (fluorescence imaging plate reader; Molecular Devices, Sunnyvale CA, USA). Influx or efflux of ions leads to

changes in the membrane potential, which can be measured in high-throughput screening in an FLIPR using suitable fluorescent dyes. However, in contrast to the patch-clamp method, it is not possible to generate voltage changes in the FLIPR. Voltage changes are, however, an essential prerequisite for the activation of hyperpolarization-activated cation channels.

### **SUMMARY OF THE INVENTION**

For the examination of the largest possible number of substances, we have developed a process that permits, among other things, high-throughput screening (HTS) for modulators of a hyperpolarization-activated cation channel.

### **BRIEF DESCRIPTION OF THE FIGURES**

Fig. 1: The figure shows the results of practicing an assay according to the invention. The change of the measured fluorescence over time is presented as the mean of, in each case, 24 wells. A and B were obtained with cells which, during dyeing, contained 10  $\mu$ M forskolin in the dyeing medium. C and D are curves of cells that did not get any forskolin.

Fig. 2: The figure shows the effect of zatebradine on the change in fluorescence over time using an assay according to the invention. Curve A shows the uninhibited change over time (only 50 mM NaCl). Curve E shows the change over time, inhibited by CsCl (50 mM NaCl + 8 mM CsCl). Curves B-D show the effect of zatebradine: B: 12.5  $\mu$ M, C: 25  $\mu$ M and D: 50  $\mu$ M.

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# DETAILED DESCRIPTION OF THE INVENTION

Abbreviations used herein are listed in Table 7 below.

The present invention provides a way to hyperpolarize cells that express a hyperpolarization-activated cation channel (i.e. to activate the hyperpolarization-activated cation channel) and to maintain this hyperpolarization of the cell, for example, until a measurement of membrane potential can be taken. Under physiological conditions, a hyperpolarization of the cell that is sufficient to activate a hyperpolarization-activated cation channel is reversed by the activity of that channel. Only when hyperpolarization can be maintained is it possible to measure, for example in an FLIPR, the depolarization of the cell caused under suitable conditions by a substance that modulates the activity of the hyperpolarization-activated cation channel.

Generally speaking, the present invention provides a process for examining hyperpolarization-activated cation channels. In the process, cells that express the hyperpolarization-activated cation channels are hyperpolarized B i.e. the hyperpolarization-activated cation channel is activated B and this hyperpolarization of the cells, which is reversed under physiological conditions by the activity of the hyperpolarization-activated cation channel, is maintained. By exclusion of extracellular sodium ions, the activated channel is unable to transport sodium ions into the cells, i.e. to depolarize the cells. If, simultaneously or even prior to the addition of the sodium ions, substances are added that modulate the activity of the hyperpolarization-activated cation channel, the depolarization is affected. For example, compared to when only sodium ions are added, depolarization is increased in the case of HCN activators (for example forskolin) and reduced in the case of HCN inhibitors (for example zatebradine = 3-[3-[[2-(3,4-dimethoxyphenyl)ethyl]methylamino]propyl]-1,3,4,5-tetrahydro-7,8-dimethoxy-2H-3-benzazepin-2-one; Reiffen et al. (1990)).

By measuring the depolarization of the cells or the changes of their membrane potential, it is possible to identify substances that modulate the activity of the hyperpolarization-activated cation channel.

In one aspect, the invention generally provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein

- a) cells which express a hyperpolarization-activated cation channel are used;
- b) the cells are hyperpolarized in the presence of a potential-sensitive fluorescent dye using an isoosmolar sodium-ion-free buffer; and
- c) the change in the membrane potential of the cells following simultaneous addition of sodium ions and the substance to be examined is detected and recorded.

Thus, in embodiments, the invention provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein the process comprises

- a) providing, in a suitable container, cells that express a hyperpolarization-activated cation channel;
- b) hyperpolarizing the cells in the presence of a potential-sensitive fluorescent dye and an isoosmolar sodium-ion-free buffer;
- c) optionally, determining the membrane potential of the cells;
- d) simultaneously adding sodium ions and a sample containing at least one substance to be tested for its ability to modulate the activity of the cation channel;
- e) determining the membrane potential of the cells;
- f) determining whether the membrane potential changed upon simultaneous addition of sodium ions and the substance(s); and
- g) optionally, recording the change in membrane potential, wherein a change in membrane potential indicates the presence of at least one substance in the sample that modulates the activity of the cation channel.

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A suitable container is any container, vessel, receptacle, etc. that can be used to hold the reagents and samples to be used in the assay. Suitable containers are disclosed in, or identifiable from, literature provided by manufacturers of equipment designed to determine membrane potentials. Such equipment is publicly available and well known to those of skill in the art.

In embodiments where step "c)" is not performed, a parallel assay, using the same strain of cells at the same concentration in the same assay composition, can be run to determine the membrane potential of the cells in the absence of the sample suspected of containing at least one substance that can modulate the activity of a cation channel.

In embodiments, the assay is a high-throughput assay.

In another aspect, the invention generally provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein

- a) cells which express a hyperpolarization-activated cation channel are used;
- b) the cells are hyperpolarized in the presence of a potential-sensitive fluorescent dye using an isoosmolar sodium-ion-free buffer;
- c) the cells are incubated with a substance to be examined; and
- d) the change in the membrane potential of the cells after addition of sodium ions is detected and recorded.

Thus, in embodiments, the invention provides a process for identifying substances that modulate the activity of hyperpolarization-activated cation channels, wherein the process comprises

- a) providing, in a suitable container, cells that express a hyperpolarization-activated cation channel;
- b) hyperpolarizing the cells in the presence of a potential-sensitive fluorescent dye and an isoosmolar sodium-ion-free buffer;
- c) optionally, determining the membrane potential of the cells;
- d) incubating the cells with a sample containing at least one substance to be tested for its ability to modulate the activity of the cation channel;
- e) optionally, determining the membrane potential of the cells;
- f) optionally, determining whether the membrane potential changed upon addition of the substance(s) to be tested;
- g) adding sodium ions;
- h) determining the membrane potential of the cells;
- i) determining whether the membrane potential changed upon addition of the sodium ions; and
- j) optionally, recording the change in membrane potential, wherein a change in membrane potential between the time before the sodium ions are added and after the sodium ions are added indicates the presence of at least one substance in the sample that modulates the activity of the cation channel.

Extracellular potassium ions can be included in the assay. In certain situations, these ions can improve the function of the hyperpolarization-activation cation channels. For example, they might be included when HCN (HAC) channels are being used in the process. Thus, in embodiments of the present invention, the isoosmolar sodium-ion-free buffer comprises potassium ions ( $K^+$ ). In embodiments, the buffer comprises potassium ions in the form of potassium chloride. In embodiments, the buffer comprises potassium ions at a concentration of at least about 0.5 mM  $K^+$ . In embodiments, the buffer comprises potassium ions at a concentration of at least about 0.8 mM  $K^+$ . In embodiments, the buffer

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comprises potassium ions at a concentration of about 2 mM. In embodiments, the buffer comprises potassium ions at a concentration of about 5 mM.

In embodiments, the isoosmolar sodium-ion-free buffer comprises at least one cation that is not able to cross the membrane in amounts that correspond to the normal extracellular sodium ion concentration. For example, the buffer can comprise choline, for example in the form of choline chloride, or NMDG (N-methyl-D-glucamine). In embodiments, the isoosmolar sodium-ion-free buffer comprises both potassium ions and at least one cation that is not able to cross the membrane in amounts that correspond to the normal extracellular sodium ion concentration.

In embodiments, the isoosmolar sodium-ion-free buffer comprises a potential-sensitive dye, for example a potential-sensitive fluorescent dye. Included among these are oxonol derivatives, such as 3-bis-barbituric acid oxonol. Thus, in embodiments, the isoosmolar sodium-ion-free buffer comprises potassium ions, at least one cation that is not able to cross the membrane in amounts that correspond to the normal extracellular sodium ion concentration, and a potential-sensitive dye.

In embodiments, the buffer comprises potential-sensitive fluorescent dyes that are suitable for examining the membrane potential of nonexcitable cells. Examples of such dyes include, but are not limited to, potential-sensitive slow-response dyes. Non-limiting examples of such potential-sensitive slow-response dyes include bis-(1,3-dibutylbarbituric acid)trimethine oxonol [DiBac<sub>4</sub>(3)], bis-(1,3-diethylthiobarbituric acid)trimethine oxonol [DiSBac<sub>2</sub>(3)] or bis-(1,3-dibutylbarbituric acid)pentamethine oxonol [DiBac<sub>4</sub>(5)]. Other known and suitable potential-sensitive dyes include, but are not limited to, fast-response dyes (for example, of the styrylpyridinium type), which are used in certain embodiments in conjunction with excitable cells, such as neurons, cardiac cells, etc. These potential-sensitive dyes react in the millisecond range and are not particularly sensitive (2-10%

fluorescence change per 100 mV potential change). Other suitable dyes include slow-response dyes of the carbocyanine type. Non-limiting examples of these slow-response dyes include diOC5(3)-3,3'-dipentylloxacarbocyanine iodide, diOC6(3)-3,3'-dihexyloxacarbocyanine iodide, etc.), JC-1 (5,5',6,6'-tetrachloro-1,1'-3,3'-tetraethylbenzimidazolecarbocyanine iodide), and rhodamine 123. In embodiments, these slow-response potential-sensitive dyes are used in studies of the membrane potential of mitochondria.

One embodiment of the invention relates to the use of the fluorescent dye from the FLIPR Membrane Potential Assay Kit (Molecular Devices, Sunnyvale, CA, USA). The fluorescence of this dye can be measured using a standard emission filter, which is transparent between about 510 and about 580 nm. In embodiments, fluorescence of this dye is measured using a filter that is transparent above about 550 nm. The manufacturer of this dye and kit disclose a number of advantages of their product, over, for example, assays based on DiBac<sub>4</sub>(3), and these advantages can be applicable to the present invention.

Some of these advantages include:

- 1) the measurement of membrane potentials with the kit is not temperature sensitive, in contrast to DiBac<sub>4</sub>(3), where the temperature has to be equilibrated prior to the actual measurement in the FLIPR;
- 2) the volume added in the FLIPR can be greater than that in the case of DiBac<sub>4</sub>(3), where usually all substances have to be added in a 10-fold concentrated form;
- 3) the measurements can be carried out much more rapidly, since the kit requires a much shorter time to reach the steady state than DiBac<sub>4</sub>(3), which usually requires between 10 and 30 minutes;
- 4) for many measurement protocols, a washing step prior to the addition of the dye is no longer required; and



- 5) the dye does not have to be present in each solution.

In embodiments of the present invention, the first two advantages are relied upon because these two advantages can be applied to assays of hyperpolarization-activated cation channels. The first two advantages can also be applied to embodiments of the invention that are directed to high-throughput screening, since screening of a large number of samples at once can be complicated and/or time consuming. For example, in embodiments where FLIPR II, which allows the measurement in 384-well plates and which is preferably employed for high-throughput screening thermostating, is used, these first two advantages can reduce the complications and time necessary to perform the assay process. In the case of poorly soluble substances, it is furthermore an advantage if they can be added to the cells in five-, three-, or even two-fold concentrated form instead of 10-fold concentrated form, as is typical with DiBac<sub>4</sub>(3).

In the processes of the present invention, cells having an elevated intracellular cAMP concentration can be used. Elevated intracellular cAMP concentrations can be achieved, for example, by adding cAMP derivatives that are able to cross the membrane. Non-limiting examples of such derivatives include dibutyryl-cAMP and 8-bromo-cAMP. As a further non-limiting example, the intracellular cAMP concentration can be increased by the addition of an adenylate cyclase activator, for example forskolin. When forskolin is used, successful results can be obtained when it is supplied in concentrations of less than about 100 µM. For example, forskolin can be used at a concentration of between about 1 µM and about 100 µM. It can also be used at concentrations less than about 1 µM. In embodiments, it is used at a concentration of about 10 µM. In principle, it is also possible to use all substances or ligands that activate adenylate cyclase by signal transduction in the cell line employed (for example ligands for b-adrenergic receptors, such as adrenalin, isoproterenol, noradrenalin, etc., if the cell has endogenous b-adrenergic receptors).

To depolarize the membrane potential,  $\text{Na}^+$  (which can be supplied in the form of NaCl, for example) is added in the FLIPR to the cells which have hyperpolarized by the sodium-ion-free buffer. In embodiments, the  $\text{Na}^+$  is added to achieve a final  $\text{Na}^+$  concentration of about 20-100 mM. In embodiments, it is added to achieve a final  $\text{Na}^+$  concentration of about 50 mM. In embodiments where the FLIPR Membrane Potential Assay Kit (Molecular Devices, Sunnyvale, CA, USA) is used, the final  $\text{Na}^+$  concentration can be about 20-100 mM. For example, it can be about 40-80 mM.

In embodiments, the invention relates to processes in which the hyperpolarization-activatable cation channel is an HCN1, HCN2, HCN3, HCN4 channel (where HAC1=HCN2, HAC2=HCN1, HAC3=HCN3 and HAC4=HCN4) or a KAT1 (=AKT) channel (hyperpolarization-activated potassium channel from *Arabidopsis thaliana*); a heteromultimer of these channels (i.e. a channel which is composed of subunits of different hyperpolarization-activated cation channels); or a chimeric hyperpolarization-activated cation channel (i.e. a synthetic channel in which individual subunits are composed of parts of different channels or hyperpolarization-activated cation channels). The hyperpolarization-activated cation channel is preferably a human hyperpolarization-activated cation channel, for example huHCN2, (SEQ ID NO. 1, SEQ ID NO. 2) or huHCN4 (SEQ ID NO. 3, SEQ ID NO. 4), or a murine hyperpolarization-activated cation channel muHCN2 (SEQ ID NO. 5, SEQ ID NO. 6). See Tables 1-6. On the amino acid level, the identity between muHCN2 and huHCN2 is 94.8%. In principle, the process is suitable for all cation channels which are activated by hyperpolarization. For example, it is suitable for HCN1-4 (or HAC1-4; see Biel et al. (1999)).

The cells can be any eukaryotic cells. For example, the cells can be mammalian cells, such as CHO or HEK293 cells. In embodiments, CHO cells or another cell line having comparably few endogenous potassium channels are used, since endogenous potassium channels might interfere with the measurement, for example, in the FLIPR. In other

embodiments cells whose endogenous potassium channels are not functionally expressed (for example the corresponding knock-out cells) are used.

The cells can, but do not necessarily, contain nucleic acids (i.e., RNA, DNA, PNA) that codes for the hyperpolarization-activated cation channel. In embodiments, the cells contain DNA. In embodiments, the cells contain RNA. In embodiments, the cells contain a cDNA of a hyperpolarization-activated cation channel in a suitable plasmid. Such cells can be prepared by transfecting the original cell line with a plasmid that contains the cDNA of a hyperpolarization-activated cation channel. Other techniques can be used as well. Techniques for introducing heterologous nucleic acids into cells are well known and widely practiced by those of skill in the art, and thus need not be detailed here.

In the case of the hyperpolarization-activated cation channels, it is an object of the invention to detect, and optionally, record changes in the membrane potential of the cells, where the changes are the result of the activation or the inhibition of these channels. Detection can utilize bis-barbituric acid oxonols. Three bis-barbituric acid oxonols (see, for example, "Handbook of Fluorescent Probes and Research Chemicals", 6th edition, Molecular Probes, Eugene OR, USA), which are mainly referred to as DiBac dyes, form a family of potential-sensitive dyes having excitation maxima at 490 nm (DiBac<sub>4</sub>(3)), 530 nm (DiSBac<sub>2</sub>(3)), and 590 nm (DiBac<sub>4</sub>(5)). The dyes get into depolarized cells by binding to intracellular proteins or membranes, leading to increased fluorescence and a red shift. Hyperpolarization results in the expulsion of the anionic dyes and thus in a decrease in fluorescence. This decrease in fluorescence can be measured, for example, with the measuring device FLIPR. Accordingly, one embodiment of the invention relates to the measurement of the membrane potential in a Fluorescent Imaging Plate Reader (FLIPR).

The FLIPR (for: Fluorescent Imaging Plate Reader; Manufacturer: Molecular Devices, Sunnyvale, CA, USA) is a measuring device that allows the simultaneous measurement

of changes of the fluorescence intensity in all wells of a microtiter plate. The dyes used are excited at about 488 nm using an argon laser, which is integrated into the system. The standard emission filter of the system is transparent in the range from 510 B 580 nm. The emitted fluorescence is registered using a CCD camera, and the system permits the simultaneous recording, within an interval of about one second, of the fluorescence in all wells of a 96-well or 384-well microtiter plate. Using a built-in pipettor, it is even possible to determine the fluorescence during the addition of the substance, which can be beneficial, for example, in the case of rapid processes. By means of special optics, the fluorescence can be registered in a layer of only about 50 mm, but not in the entire well. This can be beneficial for background reduction in all measurements where the fluorescent dye is also present extracellularly. Such a situation can exist, for example, in the measurement of changes in membrane potential using DiBac dyes. Standard applications of the system are measurements of the intracellular calcium concentration or the membrane potential of cells. Among the dyes mentioned above, DiBac<sub>4</sub>(3), which, owing to its excitation maximum, is most suitable for the argon laser in the FLIPR, has the highest sensitivity for voltage differences.

Since the DiBac<sub>4</sub>(3) takes some time to come to equilibrium, the measurement can be taken after a certain incubation time. In embodiments, the incubation temperature is at or about the optimal temperature for growth and metabolism of the biological cells being used in the assay. For example, the incubation temperature can be at or about 37°C. Incubation time can be varied to achieve complete or uniform sample temperature. In embodiments, the sample can be incubated for at least about 10 minutes. In embodiments, the sample is incubated for about or precisely 30 minutes.

Although results can be obtained at any time desired, in order to obtain as reliable of a result as possible or practical, the results should be determined and, optionally, recorded as quickly as possible after each incubation step. This is because cooling of the dye

solution might affect the result of the measurement. Thus, prior to any measurement, the composition to be measured can be incubated at a chosen temperature for a period of time that is sufficient to equilibrate the temperature of the composition at a desired level. For example, the composition can be incubated for at least about one minute, or at least about two, three, four, five, or even more minutes. Included are incubation periods prior to initial measurements (e.g., to determine base-line levels of activity or membrane potential). As with the other incubation periods, this pre-incubation phase can be carried out to compensate for temperature variations on the microtiter plate.

In embodiments where FLIPR is used, the measurement is typically carried out using the temperature parameters preset by the FLIPR manufacturer for the measurement of membrane potentials (about 37°C). However, this is a guideline, and those practicing the invention can alter the temperature to achieve maximal results. Such temperature modifications are well within the skill of those in the art, and do not represent undue experimentation. In embodiments, the parameters preset by the FLIPR manufacturer are followed essentially precisely.

Although variations in volume can be accounted for, in the FLIPR, in embodiments of the present invention, the volume of the reaction solution in which the process is carried out is changed as little as possible. In embodiments where DiBac<sub>4</sub>(3) is used, the DiBac<sub>4</sub>(3) signal is most reproducible if only relatively small volume changes take place in the FLIPR; thus, the volume is typically maintained throughout, to the extent possible and practicable. Accordingly, in these embodiments, the substances to be tested are added as concentrated solutions. In embodiments, they are added at a concentration of at least about 2-fold. For example, they can be added in about a five-fold, ten-fold, or even greater concentrated form to the DiBac<sub>4</sub>(3)-dyed cells.

Since the fluorescence measurement with the FLIPR Membrane Potential Assay Kit is not temperature-sensitive, it can be carried out simply at room temperature. This can be advantageous, for example, in embodiments that utilize the FLIPR II, which allows measurements with 384-well microtiter plates.

In embodiments, the HCN channels are activated by hyperpolarization (for example HCN2 at B100 mV to about 50%) and cause a depolarization of the cells. By increasing the intracellular cAMP concentration (for example with dibutyryl-cAMP or with forskolin), the value of the half-maximal activation can be shifted by about 10 mV to more positive potentials (Ludwig et al., 1998).

Electrophysiologically, HCN channels can be studied easily on stably transfected cells using the patch-clamp method, as voltage changes can be brought about easily. In contrast, in the FLIPR, it is not possible to induce voltage changes, and exactly because of the HCN activity, a hyperpolarization of the cells would only be transient. It has not been possible to achieve hyperpolarization of the transfected cells by adding an HCN2 inhibitor (zatebradine), since the resting membrane potential of the transfected cells is much too far removed from the potentials at which HCN2 is activated.

On the one hand, hyperpolarization is required for HCN activation. However, on the other hand, under physiological conditions, an activated HCN leads immediately to depolarization. Accordingly, in the present invention, conditions are provided under which the HCN channels can be activated by hyperpolarization, but where depolarization by the activated HCN channel is initially impossible. To this end, the cells, for example cells seeded in microtiter plates, are washed in an isoosmolar buffer in which NaCl has been replaced by another chloride salt, such as choline chloride. In embodiments, the wash buffer also contains at least some KCl, since extracellular  $K^+$  can improve HCN activation (Biel et al. 1999). In embodiments, the wash buffer contains at least 1mM KCl. In

embodiments, the wash buffer contains about 5 mM KCl. The wash buffer, which serves to effect hyperpolarization of the cation channels and thus the HCN cells, can also contain 5  $\mu$ M DiBac<sub>4</sub>(3) for measuring changes in the membrane potential in the FLIPR. By removing the extracellular Na<sup>+</sup>, the cells are hyperpolarized, i.e. the cation channel is activated. However, the HCN is not capable of causing depolarization of the cells, since the required concentration gradient of the ions Na<sup>+</sup> or K<sup>+</sup> transported by HCN is missing. Here, an activated HCN could only result in a more pronounced hyperpolarization. This is reflected in the fact that the initial fluorescence measured for HCN cells in the FLIPR at 10  $\mu$ M forskolin is lower than that without forskolin, whereas there is no difference in nontransfected cells.

In the FLIPR, Na<sup>+</sup> is added to the cells, so that the activated HCN (after a few seconds, in which there are mixing effects) causes, from about 15 seconds after the addition of Na<sup>+</sup>, depolarization of the cells, which becomes visible by an increase in fluorescence. The detection of HCN modulators can rely on a difference between cells having an activated HCN channel (e.g., only Na<sup>+</sup> addition) and cells having a blocked HCN channel (e.g., Na<sup>+</sup> + 8 mM CsCl). It has been determined that a greater difference provides a greater reliability in the system. For example, activation of the HCN channel by pre-incubation with 10  $\mu$ M forskolin increases the difference between the uninhibited 100% value from the inhibited 0% value considerably (see Fig. 1).

One embodiment of the present invention relates to the comparative determination of the change in the membrane potential of at least two cell populations incubated with different concentrations of one of the substances to be examined. In this way, the optimal concentration of the substance(s) can be determined.

Substances that are to be examined for their activity are referred to as substances to be examined or substances to be tested. Substances that are active, i.e. that modulate the

activity of the hyperpolarization-activated cation channel, can either be inhibitors (they inhibit the channel and reduce depolarization or prevent depolarization altogether) or be activators (they activate the channel and cause a more pronounced or more rapid depolarization) of the hyperpolarization-activated cation channel.

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In embodiments, the invention provides a high-throughput screening (HTS) process. In HTS, the process can be used for identifying inhibitors and/or activators of a hyperpolarization-activated cation channel. Substances identified in this manner can be used, for example, as pharmaceutically active compounds. Thus, they can be used as medicaments (medicinal compositions) or as active ingredients of medicaments.

Accordingly, the invention also provides a process that comprises the formulation of an identified substance in a pharmaceutically acceptable form. In this aspect of the invention, the methods described above can be linked to formulation of an identified substance in a pharmaceutically acceptable form. Such forms, and processes for preparing such forms, are well known to, and widely practiced by, those of skill in the art. Therefore, they need not be detailed here. Examples include, but are not limited to, forms that comprise excipients or biologically tolerable carriers.

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The invention also provides a process for preparing a medicament. The process comprises the identification of a substance that inhibits or activates the activity of a hyperpolarization-activated cation channel, and mixing the identified substance with a pharmaceutically acceptable excipient. In embodiments, the process for preparing a medicament comprises

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- a) the identification of a substance which modulates the activity of hyperpolarization-activated cation channels;
- b) the preparation of the substance;



- c) the purification of the substance; and
- d) the mixing of the substance with a pharmaceutically acceptable excipient.

The invention also provides a kit. In embodiments, the kit is a test kit for determining whether a substance modulates the activity of a hyperpolarization-activated cation channel. In embodiments, the test kit comprises

- a) cells that overexpress a hyperpolarization-activated cation channel;
- b) an isoosmolar sodium-ion-free buffer for hyperpolarizing the cell; and
- c) at least one reagent for detection of hyperpolarization activated cation channels.

The components/reagents can be those described in detail herein with respect to the assays of the invention. The components can be supplied in separate containers within the kit or in combinations within containers within the kit. Where applicable, components and/or reagents can be supplied in stabilized form. The stabilized form can permit the components and/or reagents to be maintained for extended periods of time without significant degradation or loss in activity. For example, the cells can be supplied in a cryogenic state. In addition, the salts (ions) or reagents that will comprise the assay composition can be provided in solid (dry) form, to be reconstituted with water or another appropriate solvent prior to use. Accordingly, the kit can comprise water.

As a measure for the activity of a substance, the change in the membrane potential of the cell is measured, for example, with the aid of a potential-sensitive fluorescent dye. As mentioned above, the dye can be an oxanol derivative, such as 3-bis-barbituric acid oxanol.

### Examples

The invention will now be illustrated in more detail by various examples of embodiments of the invention. The following examples are exemplary only. Thus, the scope of the invention is not limited to the embodiments disclosed in the examples. Abbreviations used in the Examples are listed in Table 7 below.

#### Example 1: Preparation of transfected cells

The plasmid pcDNA3-muHCN2 contains the murine HCN2 (muHCN2) cDNA (Genbank Accession No. AJ225122) of Pos. 22-2812 (coding sequence: Pos. 36-2627), cloned into the *EcoRI* and *NotI* cleavage sites of pcDNA3, and was obtained from M. Biel, TU Munich (Ludwig et al., 1998). In each case 6 µg of this plasmid DNA were used for transfecting CHO or HEK293 cells. For transfecting CHO cells or HEK cells, the LipofectAmine™ Reagent from Life Technologies (Gaithersburg, MD, USA) was used, in accordance with the instructions of the manufacturer. 24 hours after the transfection, the cells were transferred from culture dishes into 75 cm<sup>2</sup> cell culture bottles. 72 hours after the transfection, the cells were subjected to a selection with 400 µg/ml of the antibiotic G418 (Calbiochem, Bad Soden, Germany). Following a two-week selection, the surviving cells were detached from the bottles using trypsin-EDTA, counted in the cell counter Coulter Counter Z1 and sown into 96-well microtiter plates such that statistically, 1 cell was present per well. The microtiter plates were checked regularly under the microscope, and only cells from wells in which only one colony was growing were cultured further.

From these cells, total RNA was isolated with the aid of the QIAshredder and RNeasy kits from Qiagen (Hilden, Germany). This total RNA was examined by RT-PCR for expression of muHCN2 (Primer 1): 5'-GCCAATACCAGGAGAAG-3' [SEQ ID NO. 7], corresponds to Pos. 1354-1370 and AJ225122, and primer 2: 5'-TGAGTAGAGGCGACAGTAG-3' [SEQ ID NO. 8], corresponds to pos. 1829-1811 in AJ225122; expected RT-PCR band: 476 bp.

Example 2: Patch-clamp examination of the cells

Using the patch-clamp method, the cells with detectable mRNA expression were examined electrophysiologically, in the whole-cell configuration, for functional expression of muHCN2. This method is described in detail in Hamill et al (1981), which is incorporated herein by reference. The cells were clamped to a holding potential of -40 mV. Starting with this holding potential, the ion channels were activated by a voltage change to +140 mV for a period of one second. The current amplitude was determined the end of this pulse. Among the transfected HEK cells, some were found having currents of about 1 nA; however, owing to interfering endogenous currents, it was not possible to construct an assay for these cells in the FLIPR.

However, in the HEK cells, it was found clearly that a functionally active HCN2 channel was only detectable in cells having strong mRNA expression. In the CHO cells, the correlation between mRNA expression and function was confirmed. In general, the mRNA expression in the HEK cells was about three times better than that in the CHO cells. In the patch-clamp studies, it was possible to demonstrate a weak current in some cells of one of the most strongly expressing CHO cell lines.

Example 3: Preparation of doubly-transfected cells

Since the functional expression appeared to correlate strongly with the mRNA expression, we carried out a second transfection with the muHCN2 cDNA that had earlier been cloned into the *EcoRI* and *NotI* site of the vector pcDNA3.1(+)-zeo. After a two-week selection with G418 and Zeocin (Invitrogen, Groningen, NL), individual cell clones were isolated as described in Example 1. Following isolation of the total RNA from these cells, an RT-PCR with the primers mentioned in Example 1 was carried out. Then an RT-PCR was carried

out with the following primers, comprising a region which contains the 3'-end of the coding sequence of muHAC1 (primer 3: 5'-AGTGGCCTCGACCCACTGGACTCT-3' [SEQ ID NO. 9], corresponds to pos. 2553-2576 in AJ225122, and primer 4: 5'-CCGCCTCCTAAGCTACCTACGTCCC-3' [SEQ ID NO. 10], corresponds to pos. 2725-2701 in AJ225122).

Some of the doubly-transfected cells showed a considerably more pronounced expression both in RT-PCR and in the patch-clamp analysis than the cells which had been transfected only once. Electrophysiologically, currents of up to 11 nA were measured. These cells were used for constructing an FLIPR assay for HCN2.

#### Example 4: Construction of an FLIPR assay for HCN channels

The cells seeded on the microtiter plates are washed in an isoosmolar buffer in which NaCl has been replaced by choline chloride. However, this wash buffer also contains 5 mM KCl, since extracellular  $K^+$  is important for HCN activation (Biel et al. 1999). This wash buffer, which serves to effect hyperpolarization of the HCN cells, also contains 5  $\mu$ M DiBac<sub>4</sub>(3) for measuring changes in the membrane potential in the FLIPR. By removing the extracellular  $Na^+$ , the cells are hyperpolarized, i.e. the HCN is activated. However, the HCN is not capable of causing depolarization of the cells, since the required concentration gradient of the ions  $Na^+$  or  $K^+$  transported by HCN is missing. Here, an activated HCN could only result in a more pronounced hyperpolarization. This is reflected in the fact that the initial fluorescence measured for HCN cells in the FLIPR at 10  $\mu$ M forskolin is lower than that without forskolin, whereas there is no difference in nontransfected cells.

Since DiBac<sub>4</sub>(3) fluorescence may be sensitive to temperature variations, the measurement is, after an incubation at 37°C for 30 minutes, carried out as quickly as

possible - cooling of the dye solution may affect the measured results. Preferably, the sample is thermostated for five minutes in the FLIPR prior to the start of the measurement.

The substances to be tested are preferably added in 10-fold concentrated form to the cells which had been dyed with DiBac<sub>4</sub>(3).

In the FLIPR, Na<sup>+</sup> is added to the cells so that the activated HCN (after a few seconds, in which there are mixing effects) causes, from about 15 seconds after the addition of Na<sup>+</sup>, depolarization of the cells, which becomes visible by an increase in fluorescence. An activation of the HCN channel by preincubation with 10 μM forskolin increases the difference between the uninhibited 100% value from the inhibited 0% value considerably (see Fig. 1; discussed further below). By comparison with the control values, it can be detected whether a substance to be tested is an activator (more rapid or more pronounced depolarization) or an inhibitor (slower or inhibited depolarization, see Fig. 2: effect of zatebradine).

Figure 1 shows the change of the measured fluorescence over time, as the mean of in each case 24 wells. To be able to see the change over time between 60 and 290 seconds more clearly, the fluorescence measured in each well (sum of the brightness intensities of a fixed number of pixels, constant in all wells) at the time 60 seconds (corresponds to 40 seconds after the addition of the Na<sup>+</sup>) was defined as "0" using the FLIPR software. Curves A and B were obtained with cells which, during dyeing, contained 10 μM forskolin in the dyeing medium. C and D are curves of cells which did not get any forskolin. In A and C, NaCl was added in the FLIPR to a final concentration of 50 mM, whereas B and D additionally received 8 mM CsCl to inhibit the HCN2 channel. Using the FLIPR software, the values of curve A (with forskolin; 50 mM NaCl + 8 mM CsCl) were defined as negative controls. Curves B, C, and D represent changes in fluorescence relative to curve A. Compared to A, curve B (with forskolin; no inhibition with CsCl) shows a clear

depolarization. Compared to curve A, cells which were not preincubated with forskolin but received, simultaneously with NaCl, 8 mM CsCl (curve C) showed a decrease. This indicates that in this assay, forskolin also has an effect on the membrane potential of the cells which is independent of the HCN2 channel that is expressed. Compared to curve C, curve D (no forskolin, no inhibition with CsCl), also shows considerable depolarization. However, since the distance between curves A and B in the interval from 60 seconds onwards is always greater than the distance between curves C and D, it can be seen that forskolin activates the HCN2 channel that is expressed.

Example 5: Determination of the IC50 of an HCN2 blocker

Using the transfected HCN cells, the effect of various concentrations of the substance zatebradine, which is known as an  $I_f$  blocker, were examined (see Fig. 2; discussed further below). The inhibition by zatebradine was calculated from the relative change in fluorescence from the time 60 seconds. For each concentration of the inhibitor, the mean of in each case 6 wells of the microtiter plate was determined. From these values, the IC50 of zatebradine was calculated as 26  $\mu\text{M}$ , a value which corresponds well with the value of 31  $\mu\text{M}$  determined electrophysiologically in the same cells.

Figure 2 shows the effect of zatebradine on the change in fluorescence over time. In this figure, all cells received 10  $\mu\text{M}$  forskolin during dyeing. Curve A shows the uninhibited change over time (only 50 mM NaCl), curve E the change over time, inhibited by CsCl (50 mM NaCl + 8 mM CsCl), curves B-D show the effect of zatebradine: B: 12.5  $\mu\text{M}$ , C: 25  $\mu\text{M}$  and D: 50  $\mu\text{M}$ . Curves A and E are each means of the data from 12 wells, curves B, C and D from 6 wells. The data measured following addition of 8 mM CsCl (curve E) were defined as negative controls, so that curves A-D reflect the relative change compared to curve E. It can be seen clearly that the depolarization, which sets in after addition of

NaCl, decreases with increasing zatebradine concentration. Following export into Excel, the fluorescence data measured in the FLIPR could be used to calculate the  $IC_{50}$  for zatebradine (26  $\mu$ M).

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Example 6: Use of the FLIPR Membrane Assay Kit (Molecular Devices, Sunnyvale, USA):

Cells that were seeded a day earlier are, as before, washed three times with in each case 400  $\mu$ l of wash buffer per well. However, this time, the volume that remains above the cells after the last washing step is chosen depending on the desired  $Na^+$  and  $Cs^+$  concentrations. The dye, in wash buffer, is added, and the cells are incubated with dye for 30 minutes. The temperature is typically room temperature (about 21-25°C), but can be about 37°C.

In the FLIPR, depolarization is then induced by addition of  $Na^+$  and in some control wells inhibited again by simultaneous addition of  $Cs^+$ . Since, in the dye from Molecular Devices, an increase in the ionic strength might lead to changes in fluorescence, it has to be ensured that the ionic strength changes to the same degree in all wells of a microtiter plate. The desired final concentrations of sodium or cesium ions permitting, the osmolarity is not changed. To adjust the desired concentrations of  $Na^+$  and  $Cs^+$ , two further buffers which, instead of 140 mM of choline chloride, contain 140 mM NaCl (sodium buffer) and 140 mM CsCl (cesium buffer), respectively, are used in addition to the wash buffer.

For measurements with the FLIPR Membrane Potential Assay Kit Molecular Devices gives the following standard protocol for 96-well microtiter plates (384 wells in brackets):

On the day before the measurement, the cells are seeded in 100 ml (25 ml) of medium. Following addition of 100  $\mu$ l (25  $\mu$ l) of dye and 30 minutes of incubation at room

temperature or at 37°C, 50 µl (25 µl) of the substance to be tested, in a suitable buffer, are added in the FLIPR.

Using the volumes stated by Molecular Devices, it is possible, without changing the ionic strength, to achieve a maximum concentration of 28 mM for Na<sup>+</sup> + Cs<sup>+</sup> in 96-well plates and a maximum concentration of 46.7 mM in 384-well plates. Since this concentration, in particular in the 96-well plates, is too low for optimum activity of the hyperpolarization-activated cation channels, different volumes are tested for the individual steps.

It has been found that the dye concentrations can be reduced to half of those in the protocol given by Molecular Devices.

In 96-well plates, good results are obtained even with the following volumes: 45 µl of wash buffer supernatant above the cells, 60 µl of dye in the wash buffer, 195 µl addition volume in the FLIPR. Such a high additional volume allows a maximum concentration of Na<sup>+</sup> + Cs<sup>+</sup> of 91 mM, i.e. at 8-10 mM CsCl, the final NaCl concentration can be 81-83 mM. For 80 mM Na<sup>+</sup> and 8 mM Cs<sup>+</sup>, 6.43 µl of wash buffer, 171.43 µl of sodium buffer and 17.14 µl of cesium buffer are required, based on an added volume of 195 µl.

#### Materials and Methods:

The following materials and methods were, and can be, used to practice the invention as described in the Examples above. Other materials and methods can be used to practice other embodiments of the invention. Thus, the invention is not limited to the materials and methods disclosed below.

1. Solutions and buffers for the measurement with DiBac<sub>4</sub>(3)
- A: DiBac<sub>4</sub>(3) bis-(1,3-dibutylbarbituric acid)trimethine oxonol  
From Molecular Probes, Cat. No. B-438, MW: 516.64 g/mol



A 10 mM stock solution of DiBac<sub>4</sub>(3) is made up in DMSO (25 mg of DiBac<sub>4</sub>(3)/4.838 ml of DMSO). Aliquots of this stock solution are stored at - 20°C.

Final concentration during dyeing and addition: 5 µM.

B: Forskolin MW: 410.5 g/mol

Final concentration during dyeing: 10 µM

Aliquots of a 10 mM stock solution in DMSO are stored at - 20°C.

C: Wash buffer: (140 mM choline chloride, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1 M KOH)

D: Presoak solution for saturating the tips of the pipettes:  
as wash buffer + 10 µM DiBac<sub>4</sub>(3)

This solution is only used for the presoak plate.

E: Dye solution: double concentrated, i.e. wash buffer + 10 µM DiBac<sub>4</sub>(3) + 20 µM forskolin

F: 10-fold concentrated solution for the addition plate: 500 mM NaCl in H<sub>2</sub>O + 5 µM DiBac<sub>4</sub>(3)

All substances are made up in this solution in 10-fold concentrated form.

Positive control (final concentration): 50 mM NaCl

Negative control (final concentration): 50 mM NaCl + 8 mM CsCl

## 2. Solutions and buffers for the measurements with the FLIPR Membrane Potential Assay Kit from Molecular Devices

A: FLIPR Membrane Potential Assay Kit,  
from Molecular Probes, Cat. No. R8034

B: Wash buffer: (140 mM choline chloride, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1M KOH).

C: Dye buffer: (content of one of the "reagent vials" of the FLIPR Membrane Potential Assay Kit in 10 ml of wash buffer)

D: Sodium buffer: (140 mM NaCl, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1M KOH).

E: Cesium buffer: (140 mM CsCl, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 10 mM HEPES, 5 mM glucose, adjusted to pH 7.4 with 1M KOH).

3. Cell culture operations:

The day before the measurement, the muHCN2-transfected CHO cells are seeded at a density of 35 000 cells/well, in each case in 200  $\mu\text{l}$  of complete medium, into black 96-well microtiter plates. The cells are incubated at 37°C and 5%  $\text{CO}_2$  overnight.

4. Dyeing with DiBac<sub>4</sub>(3) and measurement in FLIPR:

Before dyeing, the cells are washed three times with 400  $\mu\text{l}$  of wash buffer in a cell washer. After the last washing step, a residual volume of 90  $\mu\text{l}$  of wash buffer/well remains above the cells.

The washed cells (with 90  $\mu\text{l}$  of wash buffer/well) are in each case incubated with 90  $\mu\text{l}$  of dye solution/well at 37°C in the  $\text{CO}_2$  incubator for 30 minutes. After this incubation time, the cell plate is measured in the FLIPR at about 37°C (preset temperature setting of the FLIPR manufacturer for measurement of membrane potentials with DiBac<sub>4</sub>(3)), either immediately or after five minutes of thermostating.

The snapshot (initial fluorescence before the start of the measurement) should on average be about 35 000 units. In the maximum, the FLIPR can resolve up to about 65 000 units.

When the program is started, the tips of the pipettes are initially saturated by immersion into presoak solution with DiBac<sub>4</sub>(3). Following this step, the actual measurement is initiated with the first measurement (t = 0 seconds). Since DiBac<sub>4</sub>(3) is a slow-response dye, it is sufficient to determine the fluorescence in the wells of the microtiter plate every 5 seconds. After about 20 seconds, the substances, which are present in the addition plate in 10-fold concentrated form, are added simultaneously to the microtiter plate using the pipettor. Since the volume after dyeing is 180 µl, 20 µl are added to each well. The measurement of the fluorescence can be terminated after about 5 minutes. For evaluation, the change in fluorescence in the interval where it is linear and in which uninhibited HCN2-transfected cells differ significantly from inhibited cells is examined.

#### 5. Dyeing with the FLIPR Membrane Potential Assay Kit and measurement in the FLIPR.

Before dyeing, the cells are washed three times with 400 µl of wash buffer in a cell washer. After the last washing step, a residual volume of 45-90 µl of wash buffer/well remains above the cells.

Following addition of the dye solution (the volume depends on the desired final concentrations), the samples are incubated at room temperature (preferred) or at 37°C in a CO<sub>2</sub> incubator for 30 minutes. Following this incubation time, the cell plate is measured at room temperature in the FLIPR.

In the FLIPR Membrane Potential Assay Kit the snapshot (initial fluorescence before the start of the measurement) may be lower than that during the measurement with DiBac<sub>4</sub>(3), since the assay kit is more sensitive to changes in the membrane potential than DiBac<sub>4</sub>(3).

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Owing to the higher achievable sensitivity, the measurement should, wherever possible (FLIPRII), be carried out using an emission filter which is transparent to light above 550 nm. However, it is also possible to carry out the measurements using the standard filter, which is transparent between 510 and 580 nm.

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When the program is started ( $t = 0$ ), the FLIPR initially determines the fluorescence of all wells of the plate a number of times, before the depolarization is started after about 20 seconds by addition of sodium ions. In each case, the addition solution is mixed from the three buffers (wash buffer, sodium buffer and cesium buffer) such that the addition results in no change of the osmolarity, or in a change which is identical in all wells. The measurement of the fluorescence can be terminated after about 5 minutes. The wells to which, in addition to  $\text{Na}^+$ , 8 mM  $\text{Cs}^+$  were added to block the HCN channel completely serve as negative control. By deducting these values from the others, a good measure for the activity of the HCN channel under the influence of the substance to be examined is obtained. For evaluation, the change in fluorescence in the interval where it is linear and in which uninhibited HCN2-transfected cells differ significantly from inhibited cells is examined.

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References:

All references disclosed herein, including the following references, are hereby incorporated herein by reference.

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Hamill O.P., Marty A., Neher E., Sakmann B., Sigworth F.J. (1981) Improved *patch-clamp* techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 391: 85-100.

Ludwig A., Zong X., Jeglitsch M., Hofmann F., Biel M. (1998) A family of hyperpolarization-activated mammalian cation channels. *Nature* 393: 587-591.

Ludwig A., Zong X., Stieber J., Hullin R., Hofmann R., Biel M. (1999) Two pacemaker channels from heart with profoundly different activation kinetics. *EMBO J.* 18: 2323-2329.

Reiffen A., Eberlein W., Müller P., Psiorz M., Noll K., Heider J., Lillie C., Kobinger W., Luger P. (1990) Specific bradycardiac agents. 1. Chemistry, pharmacology, and structure-activity relationships of substituted benzazepinones, a new class of compounds exerting antiischemic properties. *J. Med. Chem.* 33: 1496-1504.

2007-05-29

Table 1: SEQ ID NO. 1 Protein sequence of huHCN2

Accession number: AAC28444

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1 MDARGGGGRP GESPGASPTT GPPPPPPPRP PKQQPPPPPP PAPPFGPGPA PPQHPPRAEA
61 LPPEAADEGG PRGRLRSRDS SCGRPPTPGA ASTAKGSPNG ECGRGEPQCS PAGPEGPARG
121 PKVSFSCRGASGAPAGPGP AEEAGSEEAG PAGEPRGSQA SFMQRQFGAL LQPGVNKFSL
181 RMFGSQKAVE REQERVKSAG AWIIHPYSDF RFYWDFTMLL FMVGNLIIIP VGITFFKDET
241 TAPWIVFNVV SDTFFFLMDLV LNFRTGIVIE DNTEIILDPE KIKKKYLRTW FVVDFVSSIP
301 VDYIFLIVEK GIDSEVYKTA RALRIVRFTK ILSLLRLLRL SRLIRYIHQW EEIFHMTYDL
361 ASAVMRICNL ISMMLLLCHW DGCLQFLVPM LQDFPRNCWV SINGMVNHSW SELYSFALFK
421 AMSHMLCIGY GRQAPESMTD IWLTLMSMIV GATCYAMFIG HATALIQSLD SSRRQYQEKY
481 KQVEQYMSFH KLPADFRQKI HDYYEHRYQG KMFDEDSILG ELNGPLREEI VNFNCRKLVA
541 SMPLFANADP NFVTAMLTCL KFEVFQPGDY IIREGTIGKK MYFIQHGVS VLTGKNKEMK
601 LSDGSYFGEI CLLTRGRRTA SVRADTYCRL YLSVDNFNE VLEEYPMRR AFETVAIDRL
661 DRIGKKNSIL LHKVQHDLS GVFNNQENAI IQEIVKYDRE MVQQAELGQR VGLFPPPPPP
721 PQVTSATL QQAAMSFCP QVARPLVGPL ALGSPRLVRR PPPGPAPAAA SPGPPPPASP
781 PGAPASPRAP RTSPYGGPLA APLAGPALPA RRLSRASRPL SASQPSLPHG APGPAASTRP
841 ASSSTPRLGP TPAARAAAPS PDRRDSASPG AAGGLDPQDS ARSRLSSNL

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Table 2: SEQ ID NO. 2 Nucleotide sequence of huHCN2

Accession number: AF065164

1 CGGCTCCGCT CCGCACTGCC CGGCGCCGCC TCGCCATGGA CGCGCGCGGG GGCGGCGGGC  
61 GGCCCGGGGA GAGCCCGGGC GCGAGCCCA CGACCGGGCC GCGCGCGCGG CCGCCCCCGC  
121 GCGCCCCCAA ACAGCAGCCG CCGCCGCCGC CGCCGCCCGC GCGCCCCCGG GGCCCCGGGC  
181 CCGCGCCCCC CCAGCACCCG CCGCGGGCCG AGGCGTTGCC CCGGAGGCG GCGGATGAGG  
241 GCGGCGCGCG GGGCCGGCTC CGCAGCCGCG ACAGCTCGTG CGGCGCGCCC GGCACCCCGG  
301 GCGCGCGCAG CACGGCCAAG GGCAGCCCGA ACGGCGAGTG CGGCGCGCGC GAGCCGCAGT  
361 GCAGCCCCGC GGGGCCCGAG GGCOCGGCGC GGGGGCCCAA GGTGTCGTTT TCGTGCCCGC  
421 GGGCGGCCTC GGGGCCCGCG CCGGGGCCGG GCGCGCGCGA GGAGGCGGGC AGCGAGGAGG  
481 GGGGCCCGGC GGGGAGCCG CGCGGCAGCC AGGCCAGCTT CATGCAGCGC CAGTTCGGCG  
541 CGCTCCTGCA GCCGGGCGTC AACAAATTCT CGCTGCGGAT GTTCGGCAGC CAGAAGGCCG  
601 TGGAGCGCGA GCAGGAGCGC GTCAAGTCGG CGGGGGCCTG GATCATCCAC CCGTACAGCG  
661 ACTTCAGGTT CTACTGGGAC TTCACCATGC TGCTGTTTAT GGTGGGAAAC CTCATCATCA  
721 TCCCAAGTGG CATCACCTTC TTCAAGGATG AGACCACTGC CCGGTGGATC GTGTTCAACG  
781 TGGTCTCGGA CACCTTCTTC CTGATGGACC TGGTGTGAA CTTCCGCACC GGCATTGTGA  
841 TCGAGGACAA CACGAGATC ATCCTGGACC CCGAGAAGAT CAAGAAGAAG TATCTGCGCA  
901 CGTGGTTCTG GGTGGACTTC GTGTCCTCCA TCCCGTGGA CTACATCTTC CTTATCGTGG  
961 AGAAGGGCAT TGAATCCGAG GTCTACAAGA CGGCACGCGC CCTGCGCATC GTGCGCTTCA  
1021 CCAAGATCCT CAGCCTCCTG CGGCTGCTGC GCCTCTCAGC CCTGATCCGC TACATCCATC  
1081 AGTGGGAGGA GATCTTCCAC ATGACCTATG ACCTGGCCAG CGCGGTGATG AGGATCTGCA  
1141 ATCTCATCAG CATGATGCTG CTGCTCTGCC ACTGGGACGG CTGCCTGCAG TTCCTGGTGC  
1201 CTATGCTGCA GGAATTCCTG CGCAACTGCT GGGTGTCCAT CAATGGCATG GTGAACCACT  
1261 CGTGGAGTGA ACTGTACTCC TTCGCACTCT TCAAGGCCAT GAGCCACATG CTGTGCATCG  
1321 GGTACGGCCG GCAGGCGCCC GAGAGCATGA CGGACATCTG GCTGACCATG CTCAGCATGA  
1381 TTGTGGGTGC CACCTGCTAC GCCATGTTCA TCGGCCACGC CACTGCCCTC ATCCAGTCCG  
1441 TGGACTCCTC GCGGCGCCAG TACCAGGAGA AGTACAAGCA GGTGGAGCAG TACATGTCCT  
1501 TCCACAAGCT GCCAGCTGAC TTCCGCCAGA AGATCCACGA CTACTATGAG CACCGTTACC  
1561 AGGGCAAGAT GTTTGACGAG GACAGCATCC TGGGCGAGCT CAACGGGCCC CTGCGGGAGG  
1621 AGATCGTCAA CTTCAACTGC CGGAAGCTGG TGGCCTCCAT GCCGCTGTTT GCCAACGCCG  
1681 ACCCAACTT CGTCACGGCC ATGCTGACCA AGCTCAAGTT CGAGGTCTTC CAGCCGGGTG

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1741 ACTACATCAT CCGCGAAGGC ACCATCGGGA AGAAGATGTA CTTTCATCCAG CACGGCGTGG  
1801 TCAGCGTGCT CACTAAGGGC AACAAGGAGA TGAAGCTGTC CGATGGCTCC TACTTCGGGG  
1861 AGATCTGCCT GCTCACC CGGCGCCGCA CGGCGAGCGT GCGGGCTGAC ACCTACTGCC  
1921 GCCTCTATTC GCTGAGCGTG GACAACTTCA ACGAGGTGCT GGAGGAGTAC CCCATGATGC  
1981 GGCGCGCCTT CGAGACGGTG GCCATCGACC GCCTGGACCG CATCGGCAAG AAGAATTCCA  
2041 TCCTCCTGCA CAAGGTGCAG CATGACCTCA ACTCGGGCGT ATTCAACAAC CAGGAGAACG  
2101 CCATCATCCA GGAGATCGTC AAGTACGACC GCGAGATGGT GCAGCAGGCC GAGCTGGGTC  
2161 AGCGCGTGGG CCTCTTCCCG CGCGCCGCGC CGCGCCGCA GGTACCTCG GCCATCGCCA  
2221 CGCTGCAGCA GGCGCGGCC ATGAGCTTCT GCGCGCAGGT GGCGCGGCCG CTCGTGGGGC  
2281 CGCTGGCGCT CGGCTCGCG CGCCTCGTGC GCGCGCCGCG CCCGGGGCCC GCACCTGCCG  
2341 CCGCCTCACC CGGGCCCCCG CCCCCCGCA GCGCGCCGCG CCGCGCCGCG AGCCCCCGGG  
2401 CACCGCGGAC CTCGCCCTAC GGCGGCCCTGC CCGCGCCCGC CCTTGCTGGG CCGCGCCCTG  
2461 CCGCGCGCCG CCTGAGCCCG GCGTCGCGCC CACTGTCCGC CTCGCAGCCC TCGCTGCCCTC  
2521 ACGGCGCCCC CGGCCCCGCG GCCTCCACAC GCGCGGCCAG CAGCTCCACA CCGCGCTTGG  
2581 GGCCCAAGCC CGCTGCCCGG GCGCGCGCGC CCAGCCCGGA CCGCAGGGAC TCGGCCTCAC  
2641 CCGCGCGCCG CGGCGGCCG GACCCCCAGG ACTCCGCGCG CTCGCGCCTC TCGTCCAAC  
2701 TGTGACCCTC GCCGACCGCC CCGCGGGGCC AGGCGGGCCG GGGCGGGGGC CGTCATCCAG  
2761 ACCAAAGCCA TGCCATTGCG CTGCCCCGCG CGCCAGTCCG CCCAGAAGCC ATAGACGAGA  
2821 CGTAGGTAGC CGTAGTTGGA CGGACGGGCA GGGCCGGCGG GGCAGCCCCC TCCGCGCCCC  
2881 CGGCCGTCCC CCTCATCGC CCGCGGCCCA CCCCCATCGC CCTGCCCCC GCGGGCGGCC  
2941 TCGCGTGCGA GGGGGCTCCC TTCACCTCGG TGCTCAGTT CCCCCAGCTG TAAGACAGGG  
3001 ACGGGGCGGC CCAGTGCGTG AGAGGAGCCG GCTGTGGAGC CCGCGCCGCG CCGCACCTC  
3061 TAGGTGGCCC CCGTCCGAGG AGGATCGTTT TCTAAGTGCA ATACTTGGCC CGCCGGCTTC  
3121 CCGCTGCCCC CATCGCGCTC ACGCAATAAC CCGCCCGGCC CCGTCCGCG CGCGTCCCCC  
3181 GGTGACCTCG GGGAGCAGCA CCGCGCCTCC CTCCAGCACT GGCACCGAGA GGCAGGCCGTG  
3241 GCTGCGCAGG GCGCGGGGGG GAGGCTGGGG TCCCGCGGCC GTGATGAATG TACTGACGAG  
3301 CCGAGGCAGC AGTGCCCCCA CCGTGGCCCC CCACGCCCCA TTAACCCCCA CACCCCCATT  
3361 CCGCGCAATA AA

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Table 3: SEQ ID NO. 3 Protein sequence of huHCN4

Accession number: HSA132429

1 MDKLPPSMRK RLYSLPQQVG AKAWIMDEEE DAEHEGAGGR QDPSRRSIRL  
 51 RPLPSPSPSA AAGGTESRSS ALGAADSEGP ARGAGKSSTN GDCRRFRGSL  
 101 ASLGSRGGGS GGTGSGSSHG HLHDSAEERR LIAEGDASPG EDRTPPGLAA  
 151 EPERPGASAQ PAASPPPPQQ PPQPASASCE QPSVDTAIV EGGAAAGDQI  
 201 LPEAEVRLGQ AGFMQRQFGA MLQPGVKNKFS LRMFGSQKAV EREQERVKSA  
 251 GFWIIHPYSD FRFYWDLTML LLMVGNLIII PVGITFFKDE NTPPWIVFNV  
 301 VSDTFFFLIDL VLNFRGTGIV EDNTEIILDP ORIKM KYLKS WFMVDFISSI  
 351 PVDYIFLIVE TRIDSEVYKT ARALRIVRFT KILSLRLRLR LSRLIRYIHQ  
 401 WEEIFHMTYD LASAVVRIVN LIGMMLLLCH WDGCLQFLVP MLQDFPDDCW  
 451 VSINNMVNNS WGKQYSYALF KAMSHMLCIG YGRQAPVGMS DVWLTMLSMI  
 501 VGATCYAMFI GHATALIQSL DSSRRQYQEK YKQVEQYMSF HKLPPDTRQR  
 551 IHDYIEHRYQ GKMFDEESIL GELSEPLREE IINFNCRKLV ASMP LFANAD  
 601 PNFVTSMLTK LRFEVFQPGD YIIREGTIGK KMYFIQHG VV SVLTGKNKET  
 651 KLADGSYFGE ICLLTRGRRT ASVRADTYCR LYSLSVDNFN EVLEEYPMMR  
 701 RAFETVALDR LDRIGKNSI LLHKVQHD LN SGVFNYQENE IIQQIVQH DR  
 751 EMAHCAHRVQ AAASATPTPT PVIWTPLIQA PLQAAAATTS VAIALTHHPR  
 801 LPAAIFRPPP GSGLGNLGAG QTPRHLKRLQ SLIPSALGSA SPASSPSQVD  
 851 TPSSSSFHQ QLAGFSAPAG LSPLLPSSSS SPPPGACGSP SAPTPSAGVA  
 901 ATTIAGFGHF HKALGGSLS SDSPLLTPLQ PGARSPQAAQ PSPAPPGARG  
 951 GLGLPEHFLP PPPSSRSPSS SPGQLGQPPG ELSLGLATGP LSTPETPPRQ  
 1001 PEPPSLVAGA SGGASPVGFT PRGGLSPPGH SPGPPRTFPS APPRASGSHG  
 1051 SLLLPPASSP PPPQVPQRRG TPPLTPGRLT QDLKLISASQ PALPDGAQT  
 1101 LRRASPHSSG ESMAAFPLFP RAGGSGSGSG SSGGLGPPGR PYGAIPGQHV  
 1151 TLPRKTSSGS LPPPLSLFGA RATSSGGPPL TAGPQREPGA RPEPVRSKLP  
 1201 SNL\*

Table 4: SEQ ID NO. 4 Nucleotide sequence of huHCN4  
Accession number: HSA132429

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1  GGTCGCTGGG CTCCGCTCGG TTGCGGCGGG AGCCCCGGGA CGGGCCGGAC GGGCCGGGGC
61 AGAGGAGGCG AGGCGAGCTC GCGGGTGGCC AGCCACAAAG CCGGGCGGGC GAGACAGACG
121 GACAGCCAGC CCTCCCGCGG GACGCACGCC CGGGACCCGC GCGGGCCGTG CGCTCTGCAC
181 TCCGGAGCGG TTCCCTGAGC GCCGCGGCCG CAGAGCCTCT CCGGCCGGCG CCCATTGTTT
241 CCCGCGGGGG CGGGGCGCCT GGAGCCGGGG GCGCGCGCCG GCCCCTGAAC GCCAGAGGGA
301 GGGAGGGAGG CAAGAAGGGA GCGCGGGGTC CCCGCGCCCA GCGGGGCCCG GGAGGAGGTG
361 TAGCGCGGCG AGCCCGGGGA CTCGGAGCGG GACTAGGATC CTCCCCGCGG CGCGCAGCCT
421 GCCAAGCAT GGGCGCCTGA GGCTGCCCCC ACGCCGGCGG CAAAGGACGC GTCCCCACGG
481 GCGGACTGAC CGGCGGGCGG ACCTGGAGCC CGTCCGCGGC GCCGCGCTCC TGCCCCCGGC
541 CCGGTCCGAC CCCGCCCCCT GGCGCCATGG ACAAGCTGCC GCGTCCATG CGCAAGCGGC
601 TCTACAGCCT CCCGCAAGC GTGGGGGCCA AGGCGTGGAT CATGGACGAG GAAGAGGACG
661 CCGAGGAGGA GGGGGCCGGG GCGCGCCAAG ACCCCAGCCG CAGGAGCATC CGGCTCGGGC
721 CACTGCCCTC GCCCTCCCCC TCGGCGGGCG CGGGTGGCAC GGAGTCCCGG AGTCCGGCCC
781 TCGGGGCAGC GGACAGCGAA GGGCCGGCCC GCGGCGCGGG CAAGTCCAGC ACGAACGGCG
841 ACTGCAGGCG CTTCCGCGGG AGCCTGGCCT CGCTGGGCAG CCGGGGCGGC GGCAGCGGCG
901 GCACGGGGAG CGGAGCAGT CACGGACACC TGCATGACTC CGCGGAGGAG CGGCGGCTCA
961 TCGCCGAGGG CGACGCGTCC CCCGGCGAGG ACAGGACGCC CCCAGGCCTG GCGGCCGAGC
1021 CCGAGCGCCC CGGCGCCTCG GCGCAGCCCG CAGCCTCGCC GCCGCGGCCG CAGCAGCCAC
1081 CGCAGCCGGC CTCGCGCTCC TGCGAGCAGC CCTCGGTGGA CACCGCTATC AAAGTGGAGG
1141 GAGGCGCGGC TGCGGCGGAC CAGATCCTCC CGGAGGCCGA GGTGCGCCTG GGCCAGGCCG
1201 GCTTCATGCA GCGCAGTTC GGGGCCATGC TCCAACCCGG GTCAACAAA TTCTCCCTAA
1261 GGATGTTCCG CAGCCAGAAA GCCGTGGAGC GCGAACAGGA GAGGGTCAAG TCGGCCGGAT
1321 TTTGGATTAT CCACCCCTAC AGTGACTTCA GATTTTACTG GGACCTGACC ATGCTGTGTC
1381 TGATGGTGGG AAACCTGATT ATCATTCTTG TGGGCATCAC CTTCTTCAAG GATGAGAACA
1441 CCACACCTG GATTGTCTTC AATGTGGTGT CAGACACATT CTTCCTCATC GACTTGGTCC
1501 TCAACTTCCG CACAGGGATC GTGGTGGAGG ACAACACAGA GATCATCTG GACCCGACG
1561 GGATTAATAA GAAGTACCTG AAAAGCTGGT TCATGGTAGA TTTCAATTTCC TCCATCCCCG
1621 TGGACTACAT CTTCTCATC GTGGAGACAC GCATCGACTC GGAGGTCTAC AAGACTGCCC
1681 GGGCCCTGCG CATTGTCCGC TTCACGAAGA TCCTCAGCCT CTTACGCCTG TTACGCCTCT
1741 CCGCGCTCAT TCGATATATT CACCAAGTGG AAGAGATCTT CCACATGACC TACGACCTGG
1801 CCAGCGCCGT GGTGCGCATC GTGAACCTCA TCGGCATGAT GCTCCTGCTC TGCCACTGGG
1861 ACGGCTGCCT GCAGTTCCTG GTACCCATGC TACAGGACTT CCCTGACGAC TGCTGGGTGT
1921 CCATCAACAA CATGGTGAAC AACTCCTGGG GGAAGCAGTA CTCTACGCG CTCTTCAAGG
1981 CCATGAGCCA CATGCTGTGC ATCGCTACG GCGGCGAGGC GCCCGTGGGC ATGTCGACG
2041 TCTGGCTCAC CATGCTCAG ATGATCGTGG GTGCCACCTG CTACGCCATG TTCATTGGCC
2101 ACGCCACTGC CCTCATCCAG TCCCTGGACT CCTCCCGCG CAGTACCAG GAAGAATACA
2161 AGCAGGTGGA GCAGTACATG TCCTTTCACA AGCTCCCGCC CGACACCCGG CAGCGCATCC
2221 ACGACTACTA CGAGCACCGC TACCAGGGCA AGATGTTTCA CGAGGAGAGC ATCCTGGGCG
2281 AGCTAAGCGA GCCCTGCGG GAGGAGATCA TCAACTTTAA CTGTGGAAG CTGGTGGCCT
2341 CCATGCCACT GTTTGCCAAT GCGGACCCCA ACTTCGTGAC GTCCATGCTG ACCAAGCTGC
2401 GTTTCGAGGT CTTCCAGCCT GGGGACTACA TCATCCGGGA AGGCACCATT GGCAAGAAGA
2461 TGTACTTCAT CCAGCATGGC GTGGTCAGCG TGCTACCAA GGGCAACAAG GAGACCAAGC
2521 TGGCCGACGG CTCTACTTTT GGAGAGATCT GCCTGCTGAC CCGGGGCCGG CGCACAGCCA
2581 GCGTGAGGGC CGACACCTAC TGCCGCTCT ACTCGCTGAG CGTGGACAAC TTCAATGAGG
2641 TGCTGGAGGA GTACCCCATG ATGCGAAGGG CCTTCGAGAC CGTGGCGCTG GACCGCCTGG
2701 ACCGCATTGG CAAGAAGAAC TCCATCCTCC TCCACAAAGT CCAGCACGAC CTCAACTCCG
2761 GCGTCTTCAA CTCACAGGAG AATGAGATCA TCCAGCAGAT TGTGCAGCAT GACCGGGAGA
2821 TGGCCCACTG CGCGCACCGC GTCCAGGCTG CTGCCTCTGC CACCCCAACC CCCACGCCCC
2881 TCATCTGGAC CCCGCTGATC CAGGCACCAC TGCAGGCTGC CGCTGCCACC ACTTCTGTGG
2941 CCATAGCCCT CACCCACCAC CCTCGCCTGC CTGCTGCCAT CTTCGCCCCT CCCCCAGGAT
3001 CTGGGCTGGG CAACCTCGGT GCCGGGCGA CGCCAAGGCA CCTGAAACGG CTGCAGTCCC
3061 TGATCCCTTC TCGCTGGGC TCCGCTCGC CCGCCAGCAG CCCGTCCCAG GTGGACACAC
3121 CGTCTTCAAT CTCCTTCCAC ATCCAACAGC TGGGTGGATT CTCTGCCCCC CTCTGGACTGA
3181 GCCCACTCCT GCCCTCATCC AGTCTCTCCC CACCCCCCGG GGCTGTGGC TCCGCTCGG
3241 CTCCACACC ATCAGCTGGC GTAGCCGCCA CCACCATAGC CGGGTTTGGC CACTTCCACA

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3301 AGGCGCTGGG TGGCTCCCTG TCCTCCTCCG ACTCTCCCCT GCTCACCCTG CTGCAGCCAG  
3361 GCGCCCGCTC CCCGCAGGCT GCCCAGCCAT CTCCCGCGCC ACCCGGGGCC CGGGGAGGCC  
3421 TGGGACTCCC GGAGCACTTC CTGCCACCCC CACCCTCATC CAGATCCCCG TCATCTAGCC  
3481 CCGGGCAGCT GGGCCAGCCT CCGGGGAGT TGTCCCTAGG TCTGGCCACT GGCCCACTGA  
3541 GCACGCCAGA GACACCCCA CGGCAGCCTG AGCCGCCGTC CCTGTGGCA GGGGCTCTG  
3601 GGGGGGCTTC CCCTGTAGGC TTTACTCCCC GAGGAGGTCT CAGCCCCCTT GGCCACAGCC  
3661 CAGGCCCCC AAGAACCCTT CCGAGTGCCC CGCCCCGGGC CTCTGGCTCC CACGGATCCT  
3721 TGCTCCTGCC ACCTGCATCC AGCCCCCAC CACCCAGGT CCCCAGCGC CGGGGCACAC  
3781 CCCCGCTCAC CCCCGGCGC CTCACCCAGG ACCTCAAGCT CATCTCCGC TCTCAGCCAG  
3841 CCTCGCTCA GGACGGGGC CAGACTCTCC GCAGAGCCTC CCCGCACTCC TCAGGGAGT  
3901 CCATGGCTGC CTTCCCGCTC TTCCCAGGG CTGGGGGTGG CAGCGGGGGC AGTGGGAGCA  
3961 GCGGGGGCCT CGGTCCCCCT GGGAGGCCCT ATGGTGCCAT CCCCGGCCAG CACGTCACTC  
4021 TGCTCGGAA GACATCCTCA GGTTCCTTGC CACCCCTCT GTCTTTGTTT GGGGCAAGAG  
4081 CCACCTCTTC TGGGGGGCCC CCTCTGACTG CTGGACCCA GAGGGAACCT GGGGCCAGGC  
4141 CTGAGCCAGT GCGCTCCAAA CTGCCATCCA ATCTATGAGC TGGGCCCTTC CTTCCCTCTT  
4201 CTTTCTTCTT TTCTCTCCCT TCCTTCTTCC TTCAGGTTTA ACTGTGATTA GGAGATATAC  
4261 CAATAACAGT AATAATTATT TAAAAACCA CACACACCAG AAAAACAATA GACAGCAGAA  
4321 AATAACCAGG TATTCTTAGA GCTATAGATT TTTGGTCACT TGCTTTTATA GACTATTTTA  
4381 ATACTCAGCA CTAGAGGGAG GGAGGGGGAG GGAGGAGGGA GCAGGCAGGT CCCAAATGCA  
4441 AAAGCCAGAG AAAGGCAGAT GGGGTCTCCG GGGCTGGGCA GGGGTGGGAG TGGCCAGTGT  
4501 TGGCGGTTCT TAGAGCAGAT GTGTCATTGT GTTCATTAG AGAAACAGCT GCCATCAGCC  
4561 CGTTAGCTGT AACTTGGAGC TCCACTCTGC CCCCAGAAAG GGGCTGCCCT GGGGTGTGCC  
4621 CTGGGGAGCC TCAGAGCCT GCGACCTTGG GAGAAAAGGG CCAGGGCCCT GAGGGCCTAG  
4681 CATTTTTCT ACTGTAAACG TAGCAAGATC TGTATATGAA TATGTATATG TATATGTATG  
4741 TAAGATGTGT ATATGTATAG CTATGTAGCG CTCTGTAGAG CCATGTAGAT AGCCACTCAC  
4801 ATGTGCGCAC ACGTGTGCGG TCTAGTTTAA TCCCATGTTG ACAGGATGCC CAGGTCACTT  
4861 TACACCCAGC AACCCGCTT GGGCCGAGG CTGTGCACTG CATGGTCTAG GGACGTTCTC  
4921 TCTCCAGTCC TCAGGGAAGA GGACGCCAGG ACTTCGCAGC AGGCCCCCTC TCTCCCCATC  
4981 TCTGGTCTCA AAGCCAGTCC CAGCCTGACC TCTACCACA CGGAAGTGA AGACTCCCCT  
5041 TTCTAGGGC CTCAGCACA CACCG

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Table 5: SEQ ID NO. 5 Protein sequence of muHCN2

Accession number: CAA12406

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1 MDARGGGGRF GDSPGTTTAP GPPPPPPPPA PPQPQPPAP PPNPTTPSHP ESADEPGPRA
61 RLCSRDSACT PGAAGGGANG ECGRGEPQCS PEGPARGPKV SFSCRGAASG PSAAEEAGSE
121 EAGPAGEPRG SQASFLQRQF GALLQPGVNK FSLRMFGSQK AVEREQERVK SAGAWIHPY
181 SDFRFYWDFI MLLFMVGNLI IIPVGITFFK DETTAPWIVF NVVSDTFFLM DLVLNFRGTI
241 VIEDNTEIIL DPEKIKKKYL RTWFVVDVFS SIPVDYIFLI VEKGIDSEVY KTARALRIVR
301 FTKILSLRL LRLSRLIRYI HQWEEIFHMT YDLASAVMRI CNLISMMLL CHWDGCLQFL
361 VPMLQDFPSD CWSINNMMVN HSWSELYSFA LFKAMSHMLC IGYGRQAPES MTDIWLTLMS
421 MIVGATCYAM FIGHATALIQ SLDSSRRQYQ EKYKQVEQYM SFHKLPADFR QKIHDYYEHR
481 YQGMFDEDS ILGELNGPLR EEIVNFNCRK LVASMPFAN ADPNFVTAML TKLKFEVFPQ
541 GDYIIREGTI GKMYFIQHG VVSVLTGKGN EMKLSDGSYF GEICLLTRGR RTASVRADTY
601 CRLYSLSVDN FNEVLEEYPM MRRAFETVAI DRLDRIGKKN SILLHKVQHD LSSGVFNNOB
661 NAIQEIIVKY DREMVOQAEI GORVGLFPPP PPPQVTSIAI TLQQAVAMSF CPQVARPLVG
721 PLALGSPRLV RRAPPGPLPP AASPGPPAAS PPAAPSSPRA PRTSPYGVPG SPATRVGPAL
781 PARRLSRASR PLSASQPSLP HGVPAAPSPA SARPASSSTP RLGPAPTART AAPSPDRDS
841 ASPGAASGLD PLDSARSRLS SNL

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Table 6: SEQ ID NO. 6 Nucleotide sequence of muHCN2

Accession number: MMJ225122

1 CCGCTCCGCT CCGCACTGCC CGGCGCCGCC TCGCCATGGA TGCGCGCGGG GCGGCGGGGC  
61 GGCCGGGGCGA TAGTCCGGGC ACGACCCCTG CGCCGGGGCC GCGCCACCG CCGCCGCCGC  
121 CCGCGCCCCC TCAGCCTCAG CCACCACCCG CGCCACCCCG GAACCCACAG ACCCCCTCGC  
181 ACCCGGAGTC GCGGACGAG CCGGGCCCGC GCGCCCGGCT CTGCAGCCGC GACAGCGCCT  
241 GCACCCCTGG CGCGGCCAAG GCGGCGCGA ATGGCGAGTG CCGGCGCGGG GAGCCGCGT  
301 GCAGCCCCGA GGGCCCCGCG CGCGGCCCA AGGTTTCGTT CTCATGCCGC GGGGCGGCC  
361 CCGGGCCCTC GCGGGCCGAG GAGGCGGGCA GCGAGGAGGC GGGCCCGCG GGTGAGCCG  
421 GCGGCAGCCA GGCTAGCTTC CTGCAGCGCC AATTCGGGGC GCTTCTGCAG CCCGGCGTCA  
481 ACAAGTCTC CTGCGGATG TTGCGCAGCC AGAAGGCGT GGAGCGCGAG CAGGAACCG  
541 TGAAGTCGGC GGGGGCCTGG ATCATCCACC CTACAGCGA CTTAGGTTT TACTGGGACT  
601 TCACCATGCT GTTGTTCATG GTGGGAAATC TCATTATCAT TCCCGTGGG ATCACTTTCT  
661 TCAAGGACGA GACCACCGC CCTGGATCG TCTTCAACGT GGTCTCGGAC ACTTTCTTCC  
721 TCATGGACTT GGTGTGAAC TTCCGCACCG GCATTGTTAT TGAGGACAAC ACGGAGATCA  
781 TCCTGGACCC CGAGAAGATA AAGAAGAGT ACTTGCGTAC GTGGTTCGTG GTGGACTTCG  
841 TGTCATCCAT CCCGGTGGAC TACATCTTCC TCATAGTGA GAAGGGAATC GACTCCGAGG  
901 TCTACAAGAC AGCGCGTGCT CTGCGCATCG TGCGCTTAC CAAGATCCTC AGTCTGCTGC  
961 GGCTGCTGCG GCTATCACGG CTCATCCGAT ATATCCACCA GTGGGAAGAG ATTTTCCACA  
1021 TGACCTACGA CCTGGCAAGT GCAGTGATGC GCATCTGTAA CCTGATCAGC ATGATGCTAC  
1081 TGCTCTGCCA CTGGGACGGT TGCCCTGCAGT TCCTGGTGCC CATGCTGCAA GACTTCCCCA  
1141 GCGACTGCTG GGTGTCCATC AACAACATGG TGAACCACTC GTGGAGCGAG CTCTACTCGT  
1201 TCGCGCTCTT CAAGGCCATG AGCCACATGC TGTGCATCG CTACGGGCGG CAGGCGCCCC  
1261 AGAGCATGAC AGACATCTGG CTGACCATGC TCAGCATGAT CGTAGGCGCC ACCTGCTATG  
1321 CCATGTTTCT TGGGCACGCC ACTGCGCTCA TCCAGTCCCT GGATTCGTCA CGGCGCCAAT  
1381 ACCAGGAGAA GTACAAGCAA GTAGAGCAAT ACATGTCCTT CCACAACTG CCCGCTGACT  
1441 TCCGCAGAA GATCCACGAT TACTATGAAC ACCGGTACCA AGGGAAGATG TTTGATGAGG  
1501 ACAGCATCCT TGGGGAACCT AACGGGCCAC TGCGTGAGGA GATTGTGAAC TTCAACTGCC  
1561 GGAAGCTGGT GGCTTCCATG CCGCTGTTTG CCAATGCAGA CCCCACCTTC GTCACAGCCA  
1621 TGCTGACAAA GCTCAAATTT GAGGTCTTCC AGCCTGGAGA TTACATCATC CGAGAGGGGA  
1681 CCATCGGGAA GAAGATGTAC TTCATCCAGC ATGGGGTGGT GAGCGTGCTC ACCAAGGGCA  
1741 ACAAGGAGAT GAAGCTGTCG GATGGCTCCT ATTTCCGGGA GATCTGCTTG CTCACGAGGG  
1801 GCGGCGGTAC GGCCAGCGTG CGAGCTGACA CCTACTGTG CCTTACTCA CTGAGTGTGG  
1861 ACAATTTCAA CGAGGTGCTG GAGGAATACC CCATGATGCG GCGTGCCCTT GAGACTGTGG  
1921 CTATTGACCG GCTAGATCGC ATAGGCAAGA AGAATCCAT CTGCTGCAC AAGGTTTCAGC  
1981 ATGATCTCAG CTCAGGTGTG TTCAACAACC AGGAGAATGC CATCATCCAG GAGATTGTCA  
2041 AATATGACCG TGAGATGGTG CAGCAGGCAG AGCTTGCCCA GCGTGTGGGG CTCTTCCCAC  
2101 CACCGCCACC ACCGCAGGTC ACATCGGCCA TTGCCACCCT ACAGCAGGCT GTGGCCATGA  
2161 GCTTCTGCCC GCAGGTGGCC CGCCCGCTCG TGGGGCCCTT GCGGCTAGGC TCCCACGCC  
2221 TAGTGCGCCG CGCGCCCCCA GGGCCTCTGC CTCCTGCAGC CTCGCCAGGG CCACCCGCAG  
2281 CAAGCCCCCC GGCTGCACCC TCGAGCCCTC GGGCACCGCG GACCTCACCC TACGGTGTGC  
2341 CTGGCTCTCC GGCAACGCGC GTGGGGCCCG CATTGCCCGC ACGTCGCCTG AGCCGCGCCT  
2401 CGCGCCCACT GTCCGCTCG CAGCCCTCGC TGCCCATAGG CGTGCCCGCG CCCAGCCCCG  
2461 GGGCCTCTGC CGGCCCGGCC AGCAGCTCCA CGCCGCGCCT GGGACCGCA CCCACCGCCC  
2521 GGACCGCCCG GCCCAGTCCG GACCGCAGGG ACTCAGCCTC GCGGGGCGCT GCCAGTGGCC  
2581 TCGACCCACT GGACTCTGCG CGCTCGCGCC TCTCTTCCAA CTTGTGACCC TTGAGCGCCG  
2641 CCGCGCGGGC CGGGCGGGGC CGTCATCCAC ACCAAAGCCA TGCCCTCGCG CCGCCGCCCG  
2701 TGCCCGTGCA GAAGCCATAG AGGGACGTAG GTAGCTTAGG AGGCGGGCGG CCTGCGCCCC  
2761 GGCTGTCCCC CCATCGCCCT GCGCCACCCC CCATCGCCCC TGCCCCAGCG GCGGCCGCAC  
2821 GGGAGAGGGA GGGGTGCGAT CACCTCGGTG CCTCAGCCCC AACCTGGGAC AGGGACAGGG  
2881 CGGCCCTGGC CGAGGACCTG GCTGTGCCCC GCATGTGCGG TGGCCTCCGA GGAAGAATAT  
2941 GGATCAAGTG CAATACACGG CCAAGCCGGC GTGGGGGTGA GGCTGGGTCC CCGGCCGTCG  
3001 CCATGAATGT ACTGACGAGC CGAGGCAGCA GTGGCCCCCA CGCCCCATTA ACCCACAACC  
3061 CCATTCCGCG CAATAACGA CAGCATTGGC AAAAAAAAAA AA

Table 7: Abbreviations

AKT	<u>A</u> rabidopsis <u>t</u> haliana <u>K</u> <sup>+</sup> transport
cAMP	<u>c</u> yclic <u>a</u> denosine <u>m</u> onophosphate
CHO	<u>C</u> hinese <u>h</u> amster <u>o</u> vary
EDTA	<u>e</u> thylened <u>a</u> mine <u>t</u> etra <u>a</u> cetic acid
FLIPR	<u>f</u> luorescence <u>i</u> maging <u>p</u> late <u>r</u> eaders
HAC	<u>h</u> yperpolarization- <u>a</u> ctivated <u>c</u> ation channel; this name was used by some groups
HCN	<u>h</u> yperpolarization-activated <u>c</u> yclic <u>n</u> ucleotide gated cation channel; this is the new, generally accepted term
HEK	<u>h</u> uman <u>e</u> mbryonic <u>k</u> idney;
HEPES	N-2- <u>h</u> ydroxy <u>e</u> thylpiperazine-N'-2- <u>e</u> thanesulfonic acid
HTS	<u>h</u> igh- <u>t</u> hroughput <u>s</u> creening
KAT	<u>K</u> <sup>+</sup> channel from <u>A</u> rabidopsis <u>t</u> haliana

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